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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEOTIDES AND NUCLEOSIDES USING OUTERSPHERE AND INNERSPHERE METAL–SOLUTE COMPLEXES

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SUMMARY

A new bonded phase, containing a $Co(en)_3^{3+}$ moiety, was prepared in order to study the chromatographic behavior of solutes capable of forming outersphere complexes with cobalt compounds. The test solutes were nucleotides and nucleosides. It was found that solute–stationary phase interactions could be quite strong resulting in long retention times. This was particularly so with triphosphate nucleotide. To overcome this difficulty Mg(II) was added to the mobile phase. Under these conditions selectivities, efficiencies and analysis times were greatly improved.

The behavior of the $Co(en)_3^{3+}$ phase was compared to that of a C_{18} column and a diamine system. The importance of adding Mg(II) to the mobile phase is demonstrated on all systems.

INTRODUCTION

Liquid chromatography has been used extensively to separate nucleosides, nucleotides and their bases. Techniques commonly used are anion- or cation-exchange chromatography[1–5] and ligand exchange chromatography[6–9]. More recently, reversed-phase chromatography has been used for the separation of these solutes[11,10]. Isocratic separations of monophosphate, diphosphate and triphosphate nucleotides within a reasonable analysis time is difficult since their polarities vary over a wide range, and gradient elution is a common practice. However, both sensitivity and reproducibility can be limited in the gradient mode, and tedious purification of the buffer salts is sometimes necessary[12,13].

There is a need to be able to separate quickly a large number of nucleotides, nucleosides and their bases using one column and a single mobile phase or a relatively simple gradient. With this aim in mind the present work describes a new approach using a metal-ion bonded phase to accomplish a fast and selective separation of nucleotides isocratically.

Chow and Grushka[14,15] have recently demonstrated the potential of metal-ion bonded phases in high-performance liquid chromatography (HPLC). An excellent

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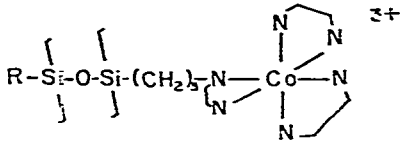
review of the utilization of metal ions in liquid chromatography was recently published by Davankov and Semechkin[16]. Previous work with metal-ion bonded phases exhibited relatively poor efficiences as evident by high height equivalent to a theoretical plate (HETP) values. This was attributed to poor mass transfer resulting from the strong solute–metal complex. To increase the rate of mass transfer several approaches are possible. Karger and co-workers[17–19] have introduced the metal ion as a chelate to the mobile phase. Alternatively the metal ion can be bonded to the support as a fully coordinated complex. Under such conditions the solute–stationary phase interactions will be via outersphere complex formation. This latter approach is described here.

The use of outersphere complexes in liquid chromatography is less common but not less impressive. Recently, Gaal and Inczecky[20] have reported the use of optical active $Co(en)_3^{3+}$ to resolve the D,L isomers of some amino acids. Bernauer[21] and Humbel *et al.*[22] have reported the resolution of some N-acetylated-α-amino acid optical isomers by the optical active complex of Fe(III) with N-(β-hydroxyethyl)-D-propylenediamine acetic acid. In these studies, however, the metal complex was adsorbed to the anion exchanger and a gradual leaching of the complex was inevitable.

The stability of the chromatographic system can be increased by bonding the metal complex to the support material. A silica bonded phase was prepared with the following Co(III) complex,



where R symbolizes the silica gel particles. $Co(en)_3^{3+}$ are known to form outersphere complexes with negatively charged species such as PO_4^{3-}, nucleotides, etc.[23–26]. Therefore, such a cobalt complex could be used as stationary phase in order to separate nucleotides.

The separation of nucleotides can be further expedited by the addition of Mg(II) ions to the mobile phase. Mg(II), which forms innersphere complexes with nucleotides[27–29] can compete with the bonded $Co(en)_3^{3+}$ for these solutes, thus improving mass transfer. The fact that the strength of the Mg–nucleotide complex depends on the number of phosphate groups should be reflected in enhanced selectivities.

It is somewhat surprising that Mg(II) is not utilized more extensively in the separation of nucleotides. Magnesium ions are co-factors in many enzymatic reactions involving nucleotides. The pH dependence of these reactions are well known. The possibility of using Mg(II) ions in an appropriate buffer as a mobile phase should prove to be a powerful approach in chromatography, even without the presence of a bonded, fully coordinated, metal cation. Experiments discussed here with a C_{18} and a diamine column show the benefits of using Mg(II) in the mobile phase. In addition such columns also allow an investigation of the importance of $Co(en)_3^{3+}$ in the separation of nucleotides.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of an Altex Model 110 solvent metering pump (Berkeley, Calif., U.S.A.), an LDC (Riviera Beach, Fla., U.S.A.), UV detector model 1285 (254 nm), and a Rheodyne injection valve (Berkeley, Calif., U.S.A.), model 7120. Columns were made of 316 stainless-steel, 20 cm × 3.1 mm I.D. for both the $Co(en)_3^{+3}$ and diamine column. A 30 cm × 4.0 mm I.D. C_{18} μBondapak column obtained from Waters Assoc. (Milford, Mass., U.S.A.) was also used.

Reagents

Partisil-10 solid support was obtained from Whatman (Clifton, N.J., U.S.A.). 3-(2-Aminoethylamino)propyltrimethoxysilane was purchased from Silar Labs (Scotia, N.Y., U.S.A.). 5'-Nucleotides and nucleosides were obtained from Sigma (St. Louis, Mo., U.S.A.). All other common chemicals were obtained from various sources. The methanol was distilled before use. The water used was distilled and deionized.

Procedure

Preparation of the diamine silica bonded phase

A 30-ml volume of 10% 3-(2-aminoethylamino)propyltrimethoxysilane in dry toluene was added to 5.0 g of Partisil-10 which was vacuum dried at 150° for 6 h. The mixture was refluxed under dry conditions for 6 h. The reacted silica gel was then washed with toluene, isopropanol, acetone, methanol and again acetone, and vacuum dried at 100° for 2 h. CHN analysis showed that 3.05 μmole/m^2 of the diamine were bonded. The surface coverage was calculated by the method suggested by Unger *et al.*[30].

Synthesis of [cis-Co(en)$_2$Cl$_2$]Cl

The synthesis was carried out as described by Bailar, Jr. [31]. The product was recrystallized before use.

Preparation of the Co(en)$_3^{3+}$ bonded phases

A 5.0-g amount of [*cis*-$Co(en)_2Cl_2$]Cl was dissolved in 100 ml of distilled water at pH 7.0. The diamine bonded silica gel (2.5 g) was added into the solution while stirring. Temperature was kept at 70°. The mixture was allowed to stir for 4 h. The following reaction occurred:

$$\begin{array}{l} \quad | \quad | \\ \text{R–Si–O–Si–(CH}_2)_3\text{NH(CH}_2)_2\text{NH}_2 + [\text{Co(en)}_2\text{Cl}_2]\text{Cl} \xrightarrow[70^\circ]{\text{pH 7}} \\ \quad | \quad | \end{array}$$

$$\begin{array}{l} \quad | \quad | \\ \text{R–Si–O–Si–(CH}_2)_3\text{–Co(en)}_3^{3+}\ (\text{Cl}^-)_3 \\ \quad | \quad | \end{array}$$

The resulting Co-bonded silica gel turned red. Since $Co(en)_3^{3+}$ crystal should be orange-red in color, it is likely that some compounds such as $Co(NH_3)H_2O(en)_2^{3+}$ or $Co(NH_3)(OH)(en)_2^{2+}$ also exist.

The silica gel was then filtered in a sintered glass funnel and washed with 200 ml of an aqueous solution having a pH of 1.5. This solution was made by

adjusting the pH of distilled water with HCl. In this manner the packing was in the Cl^- form. After further washing with 300 ml of distilled water at pH $\approx$ 7 and 200 ml methanol, the packing was vacuum dried at 70°. CHN analysis showed that 1.08 μmole/m^2 of $[Co(en)_2Cl_2]Cl$ were bonded to the support. All columns were packed in an upward direction as described by Bristow *et al.*[32].

Chromatographic studies

$Co(en)_3^{3+}$ system. The mobile phase used in all cases was a 0.037 *M* Na_2HPO_4 solution in distilled water. The pH values were adjusted with HCl. The retention behavior was studied as a function of the pH and as a function of the concentration of Mg(II) in the mobile phase. The system was equilibrated with at least 100 ml of each new mobile phase used. The amount of Mg(II) in the mobile phase was of the order of 10^{-3} *M*.

The hold-up times were measured by injecting 20 μl of a solvent having a slightly different composition than that of the mobile phases.

C_{18} system. Mobile phases contained 0.1 *M* KH_2PO_4 and various amounts of $MgSO_4 \cdot 7H_2O$. For every such mobile phase, there was a conjugate one with an equivalent amount of Na_2SO_4 instead of $MgSO_4 \cdot 7H_2O$. In such controlled systems, the effect of Mg(II) on the retention behavior can be easily identified. Concentration of Mg(II) used was in the order of $10^{-2}M$. The hold-up times were measured as mentioned before. In one study, 10% methanol was added to the mobile phase. The pH of the mobile phase was adjusted with HCl as needed.

Diamine system. A diamine column was prepared in order to isolate the effect of the $Co(en)_3^{3+}$ moiety which was bonded to a diamine group. Identical mobile phases were used with the $Co(en)_3^{3+}$ and the diamine columns.

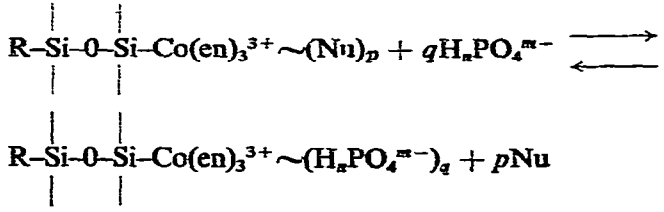
RESULTS AND DISCUSSION

$Co(en)_3^{3+}$ system

In our previous studies we have observed some leaching of the metal ions[14,15]. No evidence of such leaching was found in the present system, and the k' values were highly reproducible. Due to the large crystal field stabilization energy[33] of $Co(en)_3^{3+}$, exchange of innersphere ligands is very unlikely. This property is particularly useful to give column stability and reproducibility.

CHN analysis showed that only about 35% of the bonded diamine reacted to form the Co complex. The inaccessibility of the remaining sites might be due to steric effects, or the interactions of the amine groups with the silanol groups on the silica surface[34].

Initial attempts to separate the nucleotides were carried out with a mobile phase containing Na_2HPO_4. The chromatographic mechanism can be schematically written as

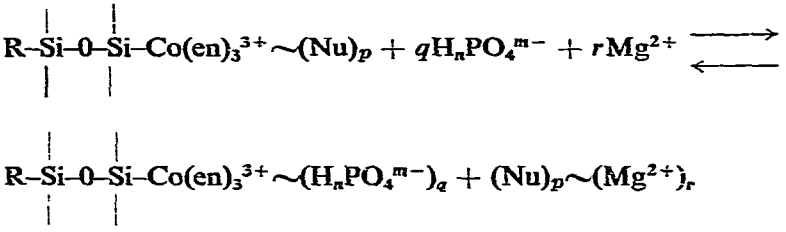


where Nu symbolizes nucleotides, m, n, q and p are integers and the wiggly line represents the interaction between the solute or the salt and the $Co(en)_3^{3+}$. For clarity the hydrocarbon backbone of the bonded phase is omitted. It was found that in order to elute the triphosphate nucleotides, mobile phases containing as much as 0.5 M Na_2HPO_4 had to be used. At such a high concentration of the phosphate salt, the selectivity for the mono- and diphosphate nucleotides was inevitably reduced.

It should be mentioned that all the nucleosides were eluted with the hold-up volume in all cases. This indicates that, as expected, the $Co(en)_3^{3+}$ bonded phase is selective for phosphate containing solutes.

Several approaches can be used in order to decrease the analysis time of the triphosphate nucleotides. The method chosen here involves adding Mg(II) ions to the mobile phase. Since magnesium ions form strong complexes with polyphosphates, the addition of Mg(II) to the mobile phase should greatly effect the retention time of the triphosphate nucleotide, decrease slightly the retention of the diphosphate nucleotides, and leave unaffected the monophosphate nucleotides.

Effect of Mg(II) in the mobile phase. With Mg(II) in the mobile phase, the separation mechanism can be looked at schematically as follows:



where r is an integer and the other symbols are as discussed above. The separation process is composed, then, of at least two competing processes: the complex formation of the $Co(en)_3^{3+}$ and of the Mg(II) with the solute. Table I clearly shows the important role of Mg(II) in the retention mechanism. Without Mg(II), the retention times of many diphosphate and triphosphate nucleotides are very long. Some triphosphates such as CTP, ATP and GTP could not be eluted. The addition of only 1×10^{-3} M Mg(II) at the pH of 6.4 changed drastically the retention times, and all the triphosphate nucleotides can now be eluted easily. Some retention order reversal is also evident; for example UTP and GDP. Less pronounced but still significant effects were observed at a pH of 5.4. Fig. 1 shows graphically the effect of the Mg(II) concentration on the retention. The behavior depicted in Fig. 1 can be easily understood from the arguments made previously regarding the stability of the tri-, di- and monophosphate nucleotides–Mg(II) complexes *vis a vis* the $Co(en)_3^{3+}$–nucleotide complexes.

The chromatogram in Fig. 2 shows the separation of a mixture consisting of one nucleoside and eight nucleotides. Under the condition shown, UTP elutes after 30 min. Fig. 3 shows a separation of a similar mixture when $1.00 \times 10^{-3}M$ Mg(II) was added to the mobile phase. The improved analysis time is obvious; UTP elutes in less than 10 min.

TABLE I

EFFECT OF THE AMOUNT OF $MgSO_4 \cdot 7H_2O$ IN THE MOBILE PHASE ON THE k' VALUES OF NUCLEOTIDES AT pH VALUES OF 6.4 AND 5.4 IN THE $Co(en)^{3+}$ SYSTEM

Solute	*2.03 × 10⁻³ M Mg(II)*		*1.20 × 10⁻³ M Mg(II)*		*1.00 × 10⁻³ M Mg(II)*		*No Mg(II)*	
	pH = 6.4	*pH = 5.4*	*pH = 6.4*	*pH = 5.4*	*pH = 6.4*	*pH = 5.4*	*pH = 6.4*	*pH = 5.4*
AMP	0.93	1.27	1.05	1.40	1.29	1.50	1.28	1.67
UMP	0.37	0.57	0.54	0.60	0.58	0.76	0.75	0.75
CMP	0.80	1.14	0.94	1.17	0.94	1.32	1.08	1.50
GMP	1.11	1.36	1.28	1.50	1.47	1.74	1.69	1.78
ADP	2.75	8.71	3.30	10.20	4.72	11.5	7.42	11.7
UDP	1.35	4.11	1.75	4.64	2.44	5.61	3.76	5.7
CDP	2.36	6.50	3.00	7.93	3.45	10.5	6.04	10.8
GDP	3.23	10.6	4.21	12.4	5.82	14.4	9.98	15.1
UTP	2.34	17.3	3.09	22.3	5.62	28.3	24.9	64.8
CTP	3.59	26.4	4.88	*	8.12	*	47.3	*
ATP	4.03	*	5.24	*	8.48	*	*	*
GTP	4.93	*	6.50	*	10.6	*	*	*

* Not eluted within reasonable time (k' values greater than 50).

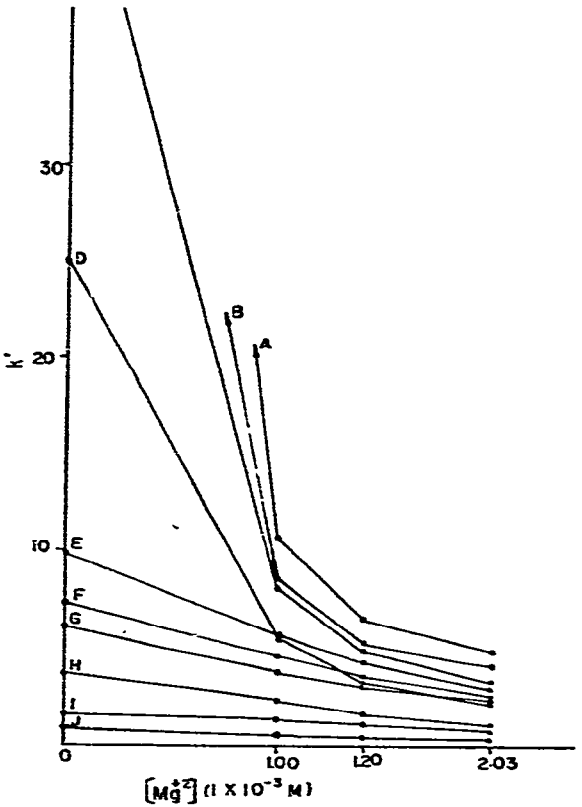


Fig. 1. The effect of the concentration of Mg(II) on the k' values of some nucleotides on the $Co(en)^{3+}$ column. Mobile phase, 0.037 *M* $Na_2HPO_4 \cdot 7H_2O$; pH = 6.4. A = GTP; B = ATP; C = CTP; D = UTP; E = GDP; F = ADP; G = CDP; H = UDP; I = GMP; J = UMP.

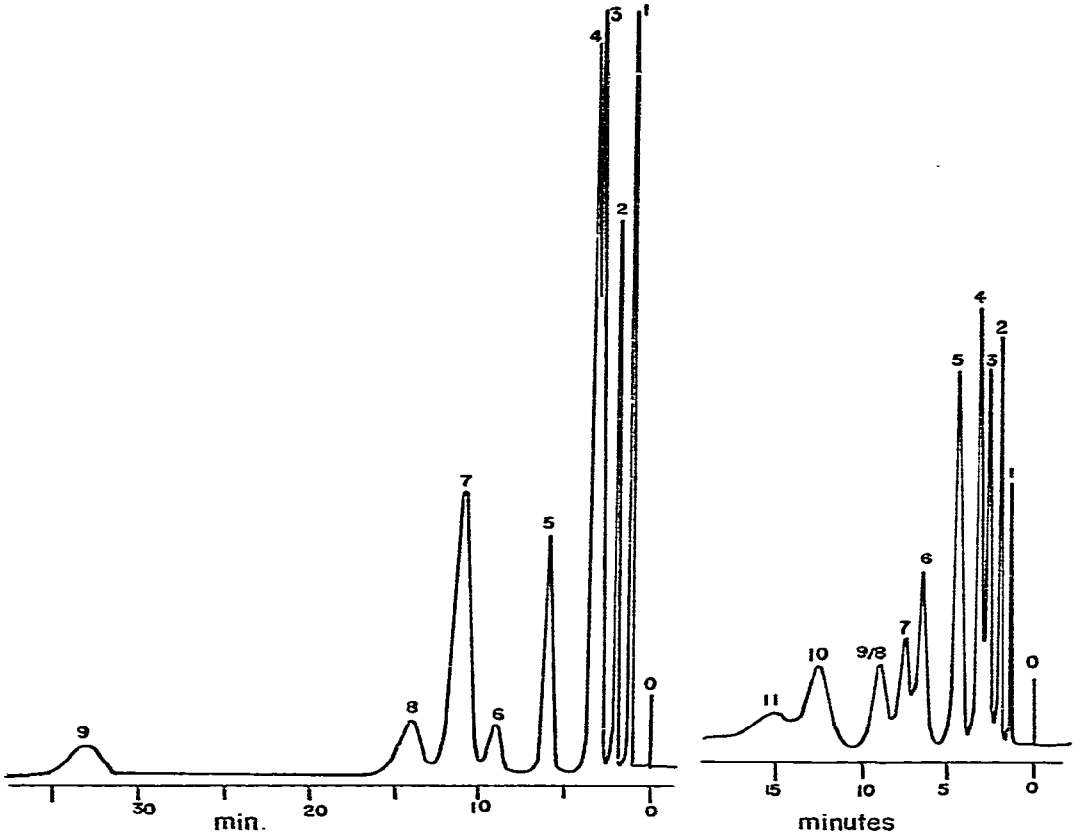


Fig. 2. Separation of some nucleotides on the $Co(en)^{3+}$ column. Mobile phase, 0.037 *M* $Na_2HPO_4 \cdot 7H_2O$; pH, 6.4; flow-rate, 1.0 ml/min. No Mg(II) in the mobile phase. 1 = Uridine; 2 = UMP; 3 = AMP; 4 = GMP; 5 = UDP; 6 = CDP; 7 = ADP; 8 = GDP; 9 = UTP.

Fig. 3. Separation of some nucleotides on the $Co(en)^{3+}$ column. Mobile phase, 0.037 *M* $Na_2HPO_4 \cdot 7H_2O$ with 1.00×10^{-3} *M* $MgSO_4 \cdot 7H_2O$; pH, 6.4; flow-rate, 1.0 ml/min. 1 = Uridine; 2 = UMP; 3 = AMP; 4 = GMP; 5 = UDP; 6 = CDP; 7 = ADP; 8 = GDP; 9 = UTP; 10 = ATP; 11 = GTP.

Effect of the mobile phase pH. Table I shows that, as expected, pH affects the retention times of the nucleotides. The effect of the pH is more clearly shown in Table II and graphically in Fig. 4. At low pH values the peaks of the di- and triphosphate nucleotides tailed badly, and some could not be eluted. On the other hand, at high pH values the nucleotide–Mg(II) complexes are quite stable and their retention times are small, resulting in incomplete separations. A mobile phase having pH of about 6.5 seems to provide the best compromise for quick analysis time and reasonable chromatographic efficiencies.

It should be mentioned again that the effect of Mg(II) concentration on the retention is small at low pH. As can be seen from Table I no retention order reversal is observed at low pH when the Mg(II) is added to the mobile phase. This, of course, is due to the dependence of the charge distribution of the nucleotides on the pH of the mobile phase.

TABLE II

EFFECT OF pH ON k' VALUES FOR $Co(en)^{3+}$ SYSTEM WITH MOBILE PHASES OF 0.037 M $Na_2HPO_4 \cdot 7H_2O$ AND 2.03×10^{-3} M $MgSO_4 \cdot 7H_2O$.

Solute	*pH = 7.00*	*pH = 6.40*	*pH = 5.50*	*pH = 4.40*
AMP	0.14	0.93	1.27	1.06
UMP	0.05	0.37	0.57	0.61
CMP	0.11	0.80	1.14	0.98
GMP	0.29	1.11	1.36	1.34
ADP	0.46	2.75	8.71	14.20
UDP	0.23	1.35	4.11	7.98
CDP	0.43	2.36	6.50	11.7
GDP	0.66	3.23	10.6	17.1
ATP	0.49	4.03	*	*
UTP	0.26	2.34	17.3	*
CTP	0.45	3.59	26.4	*
GTP	0.67	4.93	*	*

* Not eluted within reasonable time.

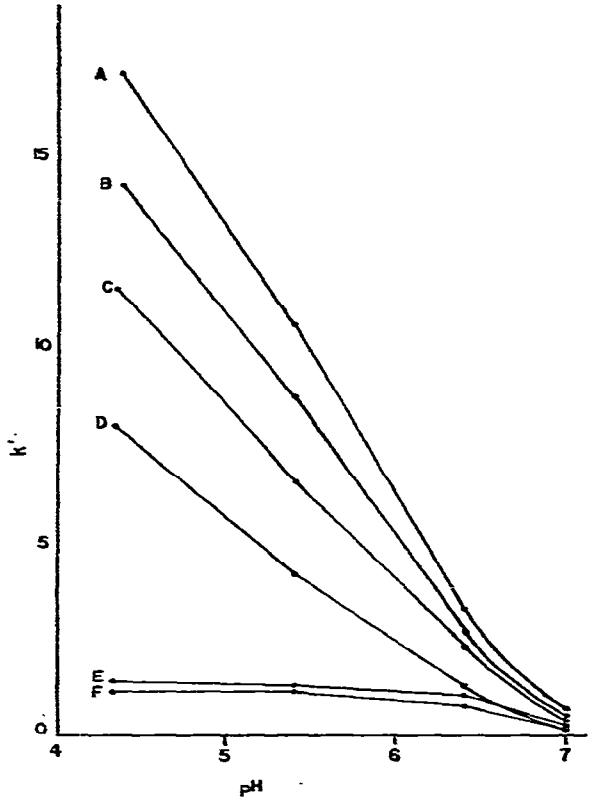


Fig. 4. Effect of the pH on the k' values of some nucleotides on the $Co(en)^{3+}$ column. Mobile phase, 0.037 M $Na_2HPO_4 \cdot 7H_2O$ with 2.03×10^{-3} M $MgSO_4 \cdot 7H_2O$ at different pH values. A = GDP; B = ADP; C = CDP; D = UDP; E = GMP; F = AMP.

Diamine system

The Co(III) complex was bonded to a diamine moiety which in turn was bonded to silica gel. It was found that only about one-third of the available diamine reacted with the Co(III) group. The effect of the underlying diamine on the retention behavior of the nucleotides should, therefore, be studied. For this purpose a diamine column was prepared and the separation of the nucleotides on it was attempted. Table III shows the results of the study. Note that while one of the mobile phases had the same composition as in Table I, the second mobile phase contained ten times as much Mg(II) ions.

TABLE III

k' VALUES ON THE DIAMINE COLUNN WITH MOBILE PHASES OF 0.037 *M* $Na_2HPO_4 \cdot 7H_2O$ WITH VARIOUS CONCENTRATIONS OF $MgSO_4 \cdot 7H_2O$ AT pH = 6.40

Solute	1.20×10^{-3} *M Mg(II)*	2.0×10^{-2} *M Mg(II)*
UMP	2.98	1.95
CMP	5.14	2.14
AMP	6.39	2.95
GMP	10.5	4.00
UDP	26.1	2.80
CDP	40.3	4.70
ADP	50.1	6.50
GDP	62.2	8.75
UTP	*	5.55
CTP	*	8.88
ATP	*	13.2
GTP	*	17.9

* Not eluted within reasonable time.

As indicated by Table III, all the triphosphate nuleotides could not be eluted within a reasonable time. Although the selectivities are quite good, the system was not efficient to give significant separations. No efforts were made to optimize the diamine system. However, the efficiency of the system can be improved by adding organic modifiers or by systematically adjusting the concentration of Mg(II) in the mobile phase.

Tables I and III clearly demonstrate the differences of the retention behavior between the $Co(en)_3^{3+}$ system and the support without Co(III). The long retention times and high selectivities on the diamine column are not unexpected. The Mg(II) ions can be complexed by the diamine group, consequently changing the column to a Mg(II) bonded phase. The separation of nucleotides, then, is governed by inner-sphere complexation with a bonded metal as described previously[14,15,17]. The triphosphate nucleotide–Mg(II) complexes are rather strong and therefore the retention times of the nucleotides are long.

In order to elute the nucleotides in reasonable times, additional Mg(II) must be added to the mobile phase. A point is reached when all the diamine sites are occupied, and the additional Mg(II) ions remain in the mobile phase. The nucleotides can be complexed by Mg(II) in the stationary phase or the mobile phase.

When the concentration of the metal ions in the mobile phase is large, complexation in the mobile phase will dominate and the retention time will decrease with further increase in the amount of Mg(II). This is the reason for the lower retention times with 2.0×10^{-2} *M* Mg(II) in the mobile phase.

It should be noted that even with high Mg(II) concentration, the retention times are longer on the diamine column than on the $Co(en)_3^{3+}$ one. In addition, the retention orders are different on both columns. There is no question that the Co(III) moiety plays a major role in controlling the separation of the nucleotides.

C_{18} system

Since Mg(II) was found to be such an effective reagent for controlling the retention times of the nucleotides in the Co(III) system, it is of interest to study its effect on the elution without the cobalt group. For such a study a reversed-phase column was chosen since here the Mg(II) cannot be complexed by the stationary phase. The use of reversed-phase for the separation of nucleotides has been studied extensively by many workers and a literature review can be found elsewhere[11].

Some of the data obtained on the reversed-phase system are shown in Table IV. The retention behavior of the nucleotides on the C_{18} column is different from that observed with the $Co(en)_3^{3+}$ column. On the latter column the retention order was monophosphate < diphosphate < triphosphate. Whereas, on the reversed-phase column, the order is pH dependent. In general, however, the retention order was found to be di < tri < monophosphate. Nucleosides could be resolved on the reversed-phase column but not on the $Co(en)_3^{3+}$ one.

TABLE IV

EFFECT OF pH ON *k'* VALUES IN THE C_{18} SYSTEM WITH MOBILE PHASES OF 0.1 *M* KH_2PO_4 WITH EITHER 0.017 *M* $MgSO_4 \cdot 7H_2O$ OR 0.017 *M* Na_2SO_4.

Solute	*pH = 4.5*		*pH = 5.0*		*pH = 5.5*		*pH = 6.0*		*pH = 6.45*	
	Mg(II)	*Na(I)*	*Mg(II)*	*Na(I)*	*Mg(II)*	*Na(I)*	*Mg(II)*	*Na(I)*	*Mg(II)*	*Na(I)*
UMP	0.96	1.03	0.68	0.94	0.80	0.81	0.72	0.82	0.69	0.82
CMP	0.71	0.71	0.50	0.61	0.50	0.42	0.42	0.52	0.42	0.49
GMP	2.38	2.37	2.01	2.35	2.16	2.51	1.63	2.69	2.04	2.29
AMP	5.90	5.92	5.88	6.50	5.88	6.10	5.19	6.12	5.24	5.08
UDP	0.54	0.39	0.42	0.48	0.31	0.45	0.30	0.40	0.38	0.46
CDP	0.36	0.39	0.21	0.30	0.23	0.28	0.19	0.27	0.25	0.30
GDP	0.95	2.46	1.61	2.46	1.90	2.72	1.16	2.66	1.20	2.08
ADP	1.79	3.85	3.19	3.89	3.12	3.82	3.10	3.78	3.20	—
UTP	0.75	1.34	0.32	0.76	0.35	0.44	0.37	—	0.45	0.41
CTP	0.54	0.93	0.25	0.52	0.23	0.40	0.21	0.36	0.22	0.29
ATP	5.25	—	3.21	6.17	3.25	5.20	3.10	4.30	3.49	3.18
GTP	3.2	*	1.66	*	1.69	*	2.40	5.96	1.03	4.12
Uridine	3.92	3.82	3.30	3.74	3.75	3.56	4.00	3.66	3.72	3.60
Cytidine	2.33	2.37	2.08	2.24	2.34	2.19	2.50	2.58	2.40	2.31
Adenosine	*	*	*	*	*	*	43.3	*	*	*
Guanosine	14.1	12.9	11.6	13.0	14.7	15.5	12.7	12.9	14.30	11.6

* Not eluted within reasonable time.

To study the effect of the Mg(II), mobile phases containing Na_2SO_4 were used (Table IV). The addition of Mg(II) to the mobile phase has the greatest effect at pH values less than 6. The retention times of the di- and triphosphates are influenced the most. It was also found that the addition of Mg(II) had changed not only the retention times and consequently the selectivity but also the efficiency. This is shown clearly in Figs. 5 and 6. The larger change in the chromatographic behavior of the di- and triphosphates as compared to the monophosphates and nucleosides should be noted. Figs. 6 and 7 clearly demonstrate the role of Mg(II) in the retention mechanism. For example, GMP and GDP, could not be resolved without Mg(II). ATP, which eluted after uridine without Mg(II), elutes before it when the metal is added to the mobile phase. The improvement in plate number, in some cases, was quite large, *e.g.* the plate number of UTP increased from 271 to 1246. This can be explained as follows. At high pH, the Mg(II)–nucleotide complexes are

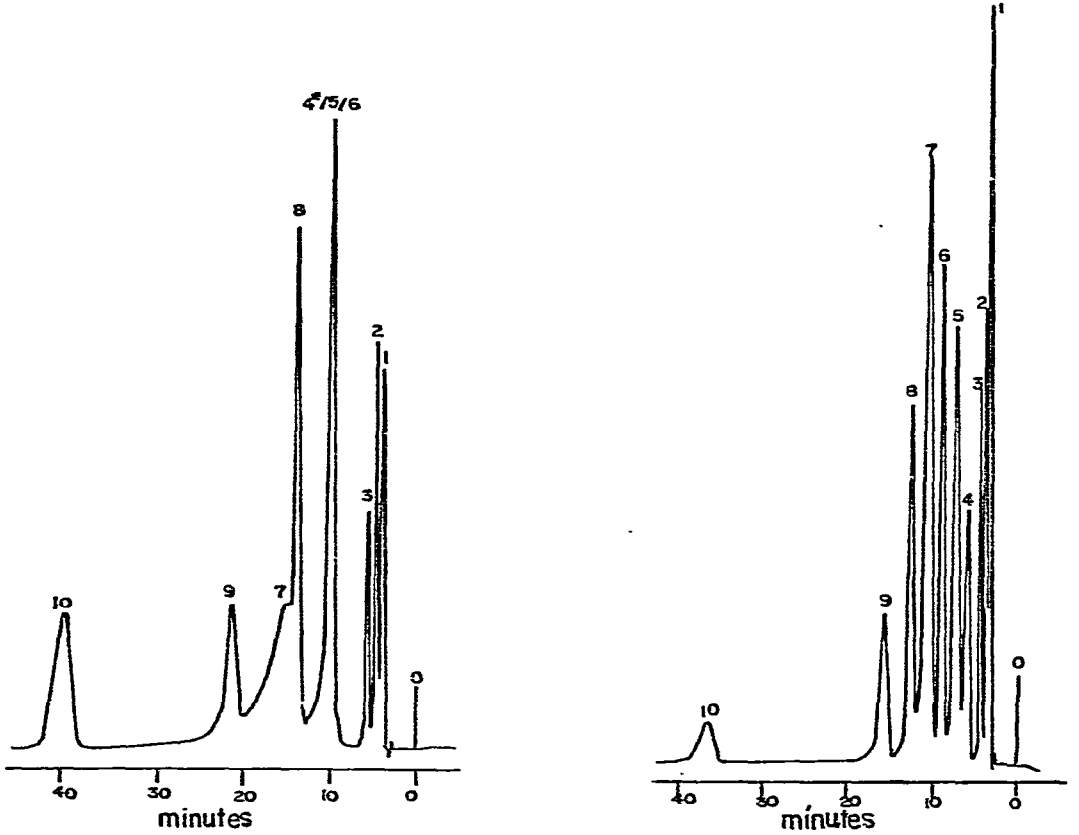


Fig. 5. Separation of some nucleosides and nucleotides on the C_{18} column. Mobile phase, 0.1 *M* KH_2PO_4 with 0.017 *M* Na_2SO_4; pH, 6.0; flow-rate, 1.1 ml/min; 1 = CDP; 2 = CMP; 3 = UMP; 4 = GDP; 5 = GMP; 6 = cytidine; 7 = ATP; 8 = uridine; 9 = AMP; 10 = guanosine.

Fig. 6. Separation of some nucleosides and nucleotides on the C_{18} column. Mobile phase, 0.1 *M* KH_2SO_4 with 0.017 *M* $MgSO_4 \cdot 7H_2O$; pH, 6.0; flow-rate, 1.0 ml/min. 1 = CDP; 2 = CMP; 3 = UMP; 4 = GDP; 5 = GMP; 6 = cytidine; 7 = ATP; 8 = uridine; 9 = AMP; 10 = guanosine.

very stable. Consequently, the solutes are relatively neutral complexes rather than the highly charged nucleotides. Neutral molecules, generally, have better efficiency in reversed-phase systems.

No attempts were made to improve the efficiency of the system. Methanol could be added to the mobile phase in order to obtain shorter analysis times and smaller HETP values.

CONCLUSION

The formation of outersphere and innersphere complexes should prove to be a promising approach to improved chromatographic selectivities. In the present study it is shown that columns containing moieties such as $Co(en)_3^{3+}$ are quite effective in separating nucleotides. The interaction between that stationary phase and the mono-, di- and triphosphate nucleotides is such that small manipulations of the mobile phase allows for the separations of a large number of solutes. The key to a successful separation in the systems studied here is the addition of a small amount of Mg(II) to the mobile phase. The fact that the stability of the Mg(II)–nucleotide complexes is dependent on the number of phosphate groups provides the analyst with a method of controlling the retention times of the nucleotides. The same effect of the Mg(II) can be observed and utilized in reversed-phase systems where outersphere complexes between the stationary phase and the solutes are not formed. In fact the reversed-phase column is more versatile since it can separate nucleotides and nucleosides. However, for the separation of nucleotides alone the $Co(en)_3^{3+}$ column might be the column of choice.

It should be emphasized that no optimization of the separation was attempted. In practice it might be more efficient to use a Mg(II) gradient elution system. Since the concentration of the Mg(II) needed to elute the strongly retained triphosphate nucleotides is small, a step gradient can be used.

The success of the $Co(en)_3^{3+}$ system should not be limited to nucleotides separation, and it could be used for the separation of many other biologically important polymers. Many of these biopolymers can form outersphere complexes with $Co(en)_3^{3+}$, and innersphere complexes with many transition metal ions. While conventional chromatographic methods have difficulties in separating biopolymers, the approach reported here can provide the necessary route to achieve such separations.

Work is now in progress to optimize the systems reported here in order to be able to separate a complex mixture of nucleosides and nucleotides.

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REFERENCES

1 J. X. Khym, *J. Chromatogr.*, 124 (1976) 415.
2 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 112 (1975) 651.
3 V. C. Runeckles and G. Das, *J. Chromatogr.*, 115 (1975) 240.

4 F. Murakami, S. Rokushika and H. Hatano, *J. Chromatogr.*, 53 (1970) 584.
5 H. J. Breter, *Anal. Biochem.*, 80 (1977) 9.
6 G. Goldstein, *Anal. Biochem.*, 20 (1967) 477.
7 C. A. Burtis and G. Goldstein, *Anal. Biochem.*, 23 (1968) 502.
8 E. Murgia, P. Richards and H. F. Walton, *J. Chromatogr.*, 87 (1973) 523.
9 K. Shimomura, L. Dickson and H. F. Walton, *Anal. Chim. Acta*, 37 (1967) 102.
10 A. M. Krstulovic, P. R. Brown and D. M. Rosie, *Anal. Chem.*, 49 (1977) 2237.
11 N. E. Hoffman and T. C. Liao, *Anal. Chem.*, 49 (1977) 2231.
12 H. W. Shmukler, *J. Chromatogr. Sci.*, 8 (1970) 653.
13 F. S. Anderson and R. C. Murphy, *J. Chromatogr.*, 121 (1976) 251.
14 F. K. Chow and E. Grushka, *Anal. Chem.*, 49 (1977) 1756.
15 F. K. Chow and E. Grushka, *Anal. Chem.*, 50 (1978) 1346.
16 V. A. Davankov and A. V. Semechkin, *J. Chromatogr.*, 141 (1977) 313.
17 N. H. C. Cooke, R. L. Viavattene, R. Eksteen, W. S. Wong, G. Davies and B. L. Karger, *J. Chromatogr.*, 149 (1978) 39.
18 B. L. Karger, W. S. Wong, R. L. Viavattene, J. N. LePage and G. Davies, *J. Chromatogr.*, 167 (1978) 253.
19 J. N. LePage, W. Lindner, G. Davies, D. E. Seitz and B. L. Karger, *Anal. Chem.*, 51 (1979) 433.
20 J. Gaal and J. Inczecky, *Talanta*, 23 (1976) 78.
21 K. Bernauer, *Swiss. Pat.*, No. 490,292 (1968).
22 H. F. Humbel, D. Vonderschmitt and K. Bernauer, *Helv. Chim. Acta*, 53 (1970) 1983.
23 M. T. Beck, *Coord. Chem. Rev.*, 3 (1968) 91.
24 L. Ilcheva, M. Georgieva, P. Bozadziev and I. Krashev, *J. Inorg. Nucl. Chem.*, 39 (1977) 1369.
25 M. Vouras and M. Schubert, *J. Amer. Chem. Soc.*, 79 (1956) 792.
26 J. R. Dunstone, *Biochem. J.*, 85 (1962) 326.
27 C. M. Frey and J. E. Stuehr, *J. Amer. Chem. Soc.*, 100 (1978) 134.
28 C. M. Frey and J. E. Stuehr, *J. Amer. Chem. Soc.*, 100 (1978) 139.
29 C. M. Frey, J. L. Banyasz and J. E. Stuehr, *J. Amer. Chem. Soc.*, 94 (1972) 9198.
30 K. K. Unger, N. Becker and P. Roumeliotis, *J. Chromatogr.*, 125 (1976) 115.
31 J. C. Bailar, Jr., in W. C. Fernelius (Editor), *Inorganic Synthesis*, Vol. II, McGraw Hill, New York, 1946, p. 222.
32 P. A. Bristow, P. N. Brittain, C. M. Riley and B. F. Williamson, *J. Chromatogr.*, 131 (1977) 57.
33 J. C. Bailar, in J. C. Bailar (Editor), *The Chemistry of the Coordination Compounds*, Reinhold, 1956, Ch. 12, p. 416.
34 J. H. Knox and A. Pryde, *J. Chromatogr.*, 112 (1975) 171.
35 G. W. Brady, *J. Chem. Phys.*, 33 (1960) 1079.
36 S. F. Mason and B. J. Norman, *J. Chem. Soc., A*, (1966) 301.